ON THE NATURE OF THE NUCLEIC ACIDS AND RNA DEPENDENT DNA POLYMERASE FROM RNA TUMOR VIRUSES AND HUMAN CELLS

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ABSTRACT

i. On the nature of the provirus. Molecular hybridization between radioactive viral RNA and DNA isolated from "uninfected" animals showed that 10-30% of the viral information was present in the cell DNA. Hybridization was observed only to DNA from the natural host. Work with RD114 virus RNA showed that the system was useful in determining the origin of a virus and indicates that at least 25% of the RD114 information originated from cat cells.

Molecular hybridization between radioactive DNA copies of viral RNA and RNA from selected tumor viruses demonstrated that there is some similarity but no identity in these nucleotide sequences, even when leukemia viruses originating from the same species were analyzed. It is concluded that either the information of these viruses originated independently or that it has evolved at an inordinate rate.

ii. On the expression of the provirus. Induction of virus from nonproducer cells by IDU is inhibited by 3'-deoxyadenosine. This inhibition is due neither to cell killing nor cytotoxicity. It is tentatively concluded that addition of poly(A) to some RNA species is a requisite for induction of oncornaviruses.

iii. RNA and RNA directed DNA polymerase in human leukemic cells. 70S poly(A)-containing RNA is found in lymphocytes in a particulate cytoplasmic fraction having a density of 1.16. An endogenous and completely RNA-dependent (i.e., RNAase-sensitive) DNA polymerase activity was also found in this fraction. The activity was obtained from leukemic blood lymphocytes (and myeloblasts) and from PHA stimulated (but not in unstimulated) normal human blood lymphocytes. Analysis of the product showed that the DNA was 3S to 5S in size and was associated with RNA. The DNA polymerase purified from the leukemic pellet (but not that from the pellet obtained from PHA stimulated normal lymphocytes) transcribed heteropolymeric portions of RLV and AMV 70S RNA. The enzyme is a true DNA polymerase and not a terminal addition enzyme by several criteria. Although this enzyme has all the biochemical characteristics of oncornavirus reverse transcriptase, its origin (viral vs. cellular) has not been determined. Immunological studies are now in progress which may help to provide an answer to this question.

INTRODUCTION

Episomes can exist in either of two freely reversible states: extrachromosomal or integrated (see Figure 4). While the episome is in its extrachromosomal state, no duplicate copies need exist in the chromosome complement. In contrast to this model, Heubner and Todaro proposed in 1969 that at least part of the information in the virus RNA genome and all of the tumorigenic information derive from the chromosomes of an "uninfected" cell, portions of the genome they termed "virogenes" and "oncogenes." For the purpose of this paper we use the more general term "provirus." Appropriate intracellular conditions, in theory, lead to the expression of these genetic elements, resulting in the formation of RNA molecules, some of which become part of mature viruses. Similar inductive events leading to the formation of C-type particles can be brought about in the laboratory

by pyrimidine analogues, such as iodo-deoxyuridine (Lowy et al., 1972; Aaronson et al. 1972).

One important feature of episome systems is that the episome may be converted from its extrachromosomal state back to the integrated state. As early as 1964, Temin presented preliminary evidence that the genomic information of RNA tumor viruses could integrate into host chromosomes (Temin, 1964). Attempts to refine the assay system, that of molecular hybridization, have led to contradictory results (Baluda and Nayak, 1970; Baluda, 1972; Rosenthal et al., 1971; Varmus et al., 1972 a and b) largely because at least some of the information exists in the presumptive uninfected cell. Recently, however, heterologous infection systems have been analyzed in more appropriate hybridization systems and whereas no viral information can be detected in these uninfected cells at least 50% of the viral information can be detected in DNA of appropriately infected cells (Harel et al., 1972). These last experiments support the notion that the extrachromosomal viral information can become a part of the host genome. Presumably, this integration requires the synthesis of DNA copies of the viral RNA via the RNA-dependent DNA polymerase associated with these viruses.

We present data here: 1) that upwards of 10-30% of the viral information exists in homologous "uninfected" cells; 2) that several sets having different information may exist in a genome, each set capable of giving rise to a different leukemia virus; 3) that the induction of this information is inhibited by an antibiotic which interferes primarily with the addition of poly(A) sequences on to RNA; and 4) that viral-like RNA and a reverse transcriptase are found in human leukemic cells.

MATERIALS AND METHODS

Nucleic acid Hybridization. Unlabelled nucleic acids were isolated according to published procedures (Gillespie and Spiegelman, 1965; Perry et al., 1972). For the preparation of radioactively labelled virus RNA, washed virus-producing cells were incubated for 8 hours in medium containing 100 μC/ml of ^{3}H-uridine. Particulate material was collected from the medium by centrifugation. RNA was isolated by phenol extraction and subjected to sucrose gradient velocity centrifugation. RNA from the 60-70S region of the gradient was used for hybridization analysis. The specific activity of the RNA was determined by measuring poly(A) content (Gillespie, Marshall, and Gallo, 1972), assuming an average of poly(A) content of 1.5%. The specific activity of the RNA preparations ranged from 5×10^5 to 1×10^6 cpm per μg of RNA, according to this assay.

Annealing of dissolved RNA to DNA immobilized on nitrocellulose filters was carried out operationally as described by Gillespie and Spiegelman (1965), using the formamide solvent system (Bonner, Kung, and Bekhov, 1967) adapted by Gillespie and Gillespie (1971). Routinely, 0.5 ng of RNA was dissolved in 100 μl of 50% formamide, 3xSSC, and 0.02 M tris, pH 7.4 (final concentrations). This solution was incubated with a nitrocellulose filter containing 50 μg of DNA at 37° in a screw cap vial. The relative amount of viral RNA to its complementary DNA would be approximately 1:1, assuming 70S RNA = 10^7 daltons, mouse genome DNA = 10^{12} daltons, and only one copy of viral information exists per cell genome. Subsequent to hybridization, filter-bound and unhybridized radioactivity were assayed as previously described (Gillespie and Gillespie, 1971). DNA on filters was assayed, after determination of radioactivity, by washing the filters twice with toluene and $CHCl_3$, releasing DNA with 1 M HCl at 60° for 45 min., then reading absorbance at 260 nm.

Annealing of dissolved radioactive DNA to RNA immobilized on phosphocellulose filters was carried out as described by Saxinger et al. (1972). RNA was attached to 6 mm filters in the 2 step system reported by these authors. Routinely, 2000 cpm of DNA product synthesized as described by Reitz et al.(1972), was dissolved in the formamide solvent system (see above) and incubated at 37° with a filter containing 25-50 ng of viral RNA or 2 μg of poly(A). Annealing was carried out

at 37° for 2 weeks, then the filters were washed with 0.01xSSC containing 0.5% SDS, dried, and counted. The amount of RNA on the filter was assayed indirectly after releasing bound DNA by measuring poly(A) bound to the filter (Gillespie, Marshall, and Gallo, 1972) and assuming a poly(A) content of viral RNA reported by these authors.

Virus and cell sources. All non-radioactive viruses were obtained from virus-producing cells grown in culture and were purchased as virus suspensions from Electro-nucleonics Inc., Bethesda, Maryland. Murine and feline leukemia viruses (MuLV, FeLV) were obtained from the following cell sources; Gross leukemia virus from AKR cells, Moloney leukemia virus from 3T3 cells, Rauscher leukemia virus from JLSV9 cells, and feline leukemia virus (Rickard strain) from a leukemic cat thymus cell suspension. Feline sarcoma virus (FeSV, Gardner strain, carrying an unidentified leukemia virus) was grown in cat embryo cells and Murine sarcoma virus (MuSV, Kirsten strain, carrying Gross leukemia virus) was grown in K3T3 cells. MuSV (Moloney) used for the preparation of radioactive RNA was grown in rat kidney cells. Mouse mammary tumor virus (MMTV) was obtained from virus producing cells explanted from a primary tumor. Mason-Pfizer monkey virus (MPMV) was obtained from Pfizer Co. Some radiolabelled 70S RNA preparations were generously donated by Dr. James East. BALB/3T3 (obtained from S. A. Aaronson) is a non-transformed non-producing cell line, BALB/K3T3 (obtained from S. A. Aaronson) is a non-producing cell line transformed by strain of MuSV (Kirsten, carrying Gross leukemia virus).

IDU induction experiments. BALB/3T3 and BALB/K3T3 were grown in modified Dulbecco's medium supplemented with 10% fetal calf serum. They were incubated at 37° with CO_2. The procedures of virus induction by IDU and reverse transcriptase assay are described elsewhere (Wu et al., 1972; see Figure 6, Table 6).

Analysis of RNA in human leukemic cells. Cells were disrupted according to Penman (1969) and a particulate cytoplasmic fraction was obtained by differential centrifugation (Sarngadharan et al., 1972; Gallo et al., 1972 b). This fraction was banded isopycnically in a 20-50% sucrose gradient. Four density regions were pooled, RNA was extracted from each fraction and exposed to velocity sedimentation in sucrose gradients, then poly(A) content of each fraction was assayed (Gillespie, Marshall, and Gallo, 1972). Full details will be reported elsewhere.

Isolation of DNA polymerases from human cells. The isolation procedures have been described in detail (Sarngadharan et al., 1972; Gallo et al., 1972 b) and are outlined in the text and in the legend to figure 7.

RESULTS

i. On the nature of proviruses

One parameter which has been used to indicate the existence of provirus in uninfected cell genomes is the presence of DNA regions in cells which are complementary in nucleotide sequence to regions of viral RNA. Conventionally, this determination is accomplished by molecular hybridization of viral RNA or its DNA copies to cell DNA. To extract maximum information from this system one should ascertain that a minor contaminant (non-viral) in the RNA preparation is not being analyzed and that the bulk of the viral information is assayed. Additionally it is desirable to analyze both repeated and unique sequences in the cell genome (Britten and Kohne, 1968) and to determine the degree of nucleotide sequence homology between the viral RNA and the cell DNA.

The first data to indicate that the DNA genome of uninfected cells carry counterparts to viral RNA information came from the experiments of Baluda and Nayak (1970), Rosenthal et al. (1971), and Baluda (1972). In these experiments it was demonstrated that 70S RNA, isolated from RNA tumor viruses, can form a hybrid structure with DNA from cells which ostensibly carry no virus. However, only about 0.1% of the input RNA was recovered in a hybrid structure and those experiments were carried out in the presence of an excess of cellular RNA to eliminate hybrids between DNA and cellular RNA which might contaminate the viral

RNA preparation. Nevertheless, interpretation of these data might be confounded if the labelled virus RNA is contaminated with extraneous labelled RNA or if virus-like RNA exists in normal cells.

We have analyzed the hybridization of radioactive 70S RNA from murine mammary tumor virus (MMTV) to DNA purified from spleens of C57 black mice. These experiments have been done under conditions where viral DNA copies would be present in roughly equal weight with viral RNA, if 1 copy per genome existed (see Materials and Methods). The formamide hybridization system using DNA immobilized on nitrocellulose membrane filters was employed to permit extended annealing experiments without DNA renaturation and to permit analysis of nuclease-sensitive complex as well as nuclease-resistant hybrid.

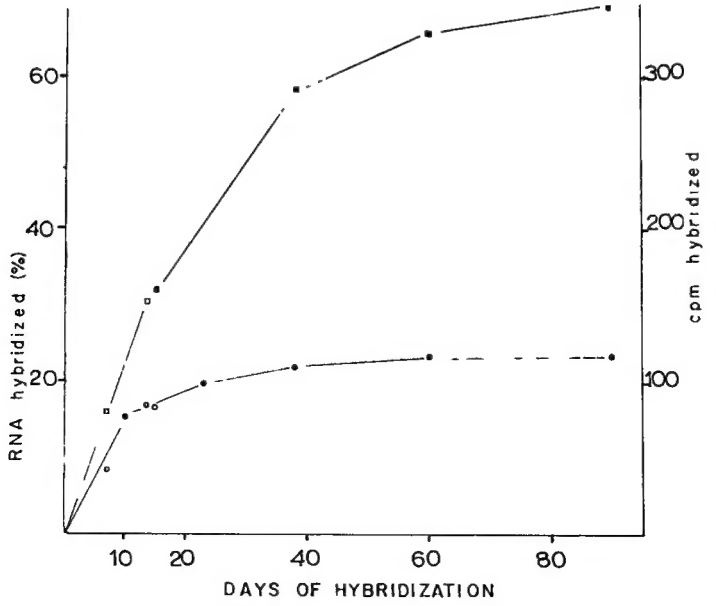

Fig. 1. Kinetics of hybridization of ^{3}H MMTV RNA to C57 mouse spleen DNA. Annealing and hybrid purification was carried out as described in Materials and Methods and Table 1. Noise levels (radioactivity attached to blank filters) was less than 1 cpm for ribonuclease treated samples (excluding machine background). Circles = samples processed with RNAase; squares = no ribonuclease. Open, closed and half-closed symbols signify separate experiments.

Figure 1 presents the kinetics of hybridization in such a system. Though complete exhaustive hybridization of the RNA has not been yet realized, 25% of the input MMTV RNA formed a ribonuclease-resistant structure with mouse spleen DNA after 90 days of hybridization. In the absence of ribonuclease treatment, about 70% of the input RNA was recovered on the DNA filter. Neither the ribonuclease-sensitive structure nor the ribonuclease-resistant hybrid results from an interaction between poly(A) in the virus RNA and poly dT in the DNA, since large amounts of unlabelled poly(A) do not affect the observed annealing (Table 1). In this particular instance it is unlikely that the hybridization response arises from contaminating cellular RNA in the MMTV RNA preparation, since a homogeneous peak of 70S RNA was recovered from the virus preparation (Figure 2).

Table 1
Effect of poly(A) on Hybridization of MMTV ^{3}H 70S RNA to Mouse or Human DNA

	poly(A) (5 µg)	RNAase	RNA hybridized (%) Mouse	Human	Poly(A)
1	-	-	28	4	0
2	-	+	13	2	0
3	+	-	28	4	0
4	+	+	18	0	0

Annealing was carried out at 37° for 14 days, using 0.5 ng (500 cpm) of RNA and 50 µg of DNA immobilized on nitrocellulose membrane filters. See Materials and Methods for procedural details. Mouse DNA was purified from spleens of C57 mice; human DNA was prepared from lymphocytes donated by a patient with leukemia.

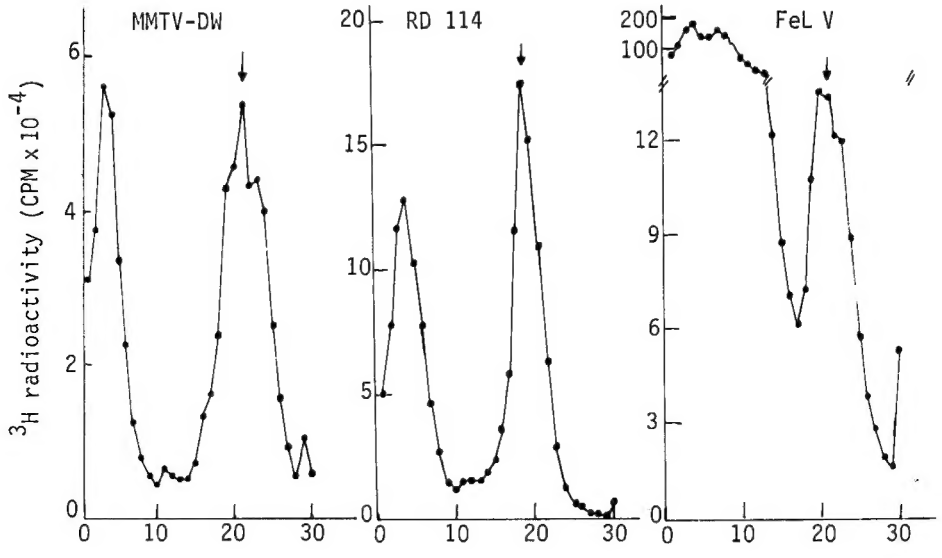

Fig. 2. Sucrose gradient preparation of 70S viral RNA. Sedimentation is from left to right; the arrow denotes the 60-70S region of the gradient. 18 and 28S marker RNA were recovered at effluent volumes of 8 and 11 ml, respectively. The 60-70S peak region was pooled and used in hybridization experiments. These experiments indicate that a minimum of 25% of the 70S MMTV RNA is represented by identical or nearly identical information in the C57 mouse genome. Experiments to be reported elsewhere, using annealing in homogeneous phase solution have shown that the viral RNA hybridizes to a DNA component which has a Cot of approximately 100 (Britten and Kohne, 1968) and that there exist 50-100 copies of these DNA sequences per haploid genome in the "uninfected" cells of the natural host. The hybrid structure appears to be perfect over at least 40 consecutive nucleotides since it resists RNAase treatment at 60° in 2xSSC (Gillespie, 1968).

Experiments with RNA from other viruses is presented in Table 2. In every case significant hybridization to homologous cellular DNA was observed, with the percent of the input RNA hybridized ranging between 12 and 30. These values are 2 orders of magnitude higher than previously reported values utilizing RNA-DNA hybridization (Baluda and Nayak, 1970; Baluda, 1972; Rosenthal et al., 1971) and

Table 2
Hybridization of 70S Viral RNA to homologous cellular DNA

RNA	RNA Hybridized (%) +RNAase	-RNAase
MMTV	36	71
MuSV	16	31
MuLV	17	32
FeLV	16	28

Annealing was carried out at 36° for 30 days, using 0.25-0.5 ng of RNA and 50 µg of DNA immobilized on nitrocellulose filters. MMTV, MuSV (Moloney), and MuLV (Rauscher) were annealed to DNA isolated from spleens of C57 mice; FeLV (Rickard) RNA was hybridized to DNA purified from "normal" cat spleens. Values are expressed as percent of the input RNA recovered in a hybrid structure.

demonstrated leukemia and mammary tumor provirus genes in mouse, and leukemia provirus in the cat systems.

Table 3
Hybridization of 70S viral RNA to Heterologous Cellular DNA

RNA	RNAase	DNA Mouse	Cat	Human
MMTV	+	31	2	0
RD114	+	3	25	1
FeLV	+	3	14	3
MMTV	-	65	0	0
RD114	-	1	40	0
FeLV	-	7	24	6

Hybridization was carried out as described in Materials and Methods and legend to Table 2. Human DNA was isolated from peripheral blood lymphocytes of a leukemic donor. Values are expressed as percent of the input RNA recovered as a hybrid structure.

The viral-specific genes detected in our assay differ in different species of animals and hence may be useful in determining the origin of a particular RNA tumor virus. Table 3 presents the hybridization of RNA from three different tumor viruses to DNA from mice, cats, or humans. MMTV RNA hybridizes only to mouse DNA and not to cat or human DNA. RNA isolated from feline leukemia virus (FeLV) hybridizes preferentially to cat DNA. The low cross-reaction between FeLV and both mouse and human DNA may be characteristic of certain viral genomes, or may reflect the relative impurity of the FeLV RNA preparation (Figure 2). Specificity of hybridization has been reported previously for the avian virus, Rous Sarcoma Virus (Rosenthal et al., 1971; Harel et al., 1972). Note from Table 3 that when RNAase is omitted from the assay an increase in sensitivity is achieved with no apparent loss in specificity.

Given the specificity of this hybridization assay, it was of interest to analyze the RNA of a virus of unknown origin. RD114 is such a virus. It was isolated by passage of human rhabdomyosarcoma cells through a cat, then propagated in the human cell line (McAllister et al., 1972). It can be seen from Table 3 that the RNA isolated from this virus anneals

only to cat DNA, among those tested. It must be concluded, therefore, that at least part of the RD114 RNA information (probably repetitive) originated from a cat cell.

It is of some interest to know whether different viruses derived from the same species of natural host arise from the same or from different genetic information. Preliminary evidence concerning this question can be obtained by examining the identity or nonidentity of information among viruses, assuming that an inordinate rate of mutation or RNA mixing does not obscure the results. For these experiments, radioactive DNA copies of viral RNA were synthesized in vitro, then annealed to a variety of RNA preparations. Hybridizations were carried out in homogeneous solution or in a 2-phase system with RNA immobilized on phosphocellulose discs (Saxinger et al., 1972). Hybrids formed in solution were assayed either by isopycnic centrifugation in Cs_2SO_4 density gradients or by resistance to digestion with a nuclease specific for single stranded nucleic acids, Aspergillus S1 nuclease (Varmus et al., 1972, a and b).

Table 4
Hybridization of DNA Products to Immobilized 70S Virus RNA

DNA	RNA								
	MuSV	MuLV (Rauscher)	FeLV	FeSV	AMV	MMTV	MPMV	VISNA	Poly (A)
MuSV-A	689	81	51	31	0	0		0	16
MuLV	2	231	0	7	0	0	0	0	15
FeLV-A	4	0	85	81	0	0	4	0	53
FeSV	2	0	35	40					
AMV		0	2		78		0	0	0
MPMV-A					0	0	320		0
VISNA-A	0	33			30	3	2	314	42

Hybridizations were carried out with RNA immobilized on phosphocellulose discs (Saxinger et al., 1972). Hybridization mixtures (100 µl) consisted of 50 µl of formamide and 50 µl of 6xSSC containing 2000 cpm of DNA product. Incubation was carried out at 36° for 14 days, then washed with 1/100 SSC containing 0.5% SDS (Reitz et al., 1972). Hybridization with visna virus DNA product was carried out at room temperature. DNA products were synthesized in endogenous reaction (Wu et al., 1972, a); those denoted with an "-A" were made in the presence of 100 µg/ml of actinomycin D. All values were corrected for radioactivity observed with blank filters (35-40 cpm. including machine background). Values = cpm hybridized.

Typical results with the immobilized RNA method are presented in Table 4. By and large, DNA products are complementary only to the RNA on which they were synthesized. Many of the results confirm earlier findings; no genetic identity was observed between AMV or MMTV and any other virus. Other results present new information. No identity was observed between strains of leukemia viruses from feline versus murine sources. It was considered that the manner of synthesis of the endogenous DNA product might influence the results, but varying the labelled nucleotide, the nucleotide concentrations, the duration of the reaction (from 12 min. to 18 hrs.) or the presence or absence of actinomycin D, did not yield a cross-reacting product. It therefore appears that "leukemia information" is not conserved from cat to mouse, at least not in the nucleotide sequences contained in the DNA products studied.

Table 5
Hybridization of MuSV DNA product to MuLV RNAs Assayed by Resistance to Aspergillus Nuclease

Sample number	RNA source	cpm
1	MuSV (Kirsten-Gross)	3350
2	MuLV (AKR, Gross)	1950
3	MuLV (Rauscher)	500
4	MuLV (Moloney)	475

MuSV DNA product was synthesized in a 45 minute endogenous reaction in the presence of actinomycin D (Wu et al., 1972, a). Annealing mixtures consisted of 7000 cpm of DNA product and 2 µg of RNA (where indicated) in 100 µl of 50% formamide (Materials and Methods). Hybridization was carried out at 36° for 6 days. Reaction products were digested with Aspergillus S1 nuclease and nuclease-resistant hybrid was measured, after precipitation with trichloroacetic acid. Nuclease-resistance of the DNA incubated alone = 1140 cpm.

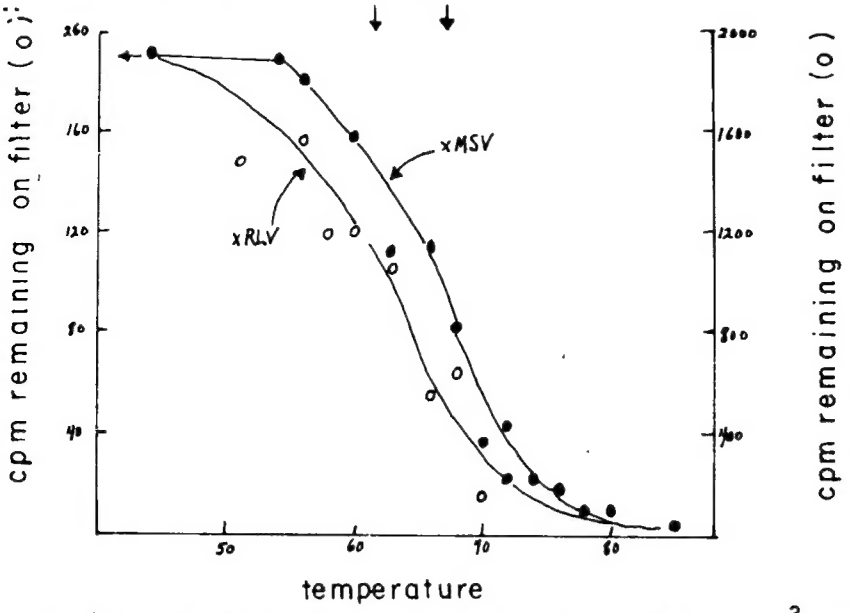

Fig. 3. Tm of homologous and heterologous hybrids formed between ^{3}H DNA and immobilized RNA. Hybridization was carried out between MSV-A DNA and MSV or RLV RNA as described in the Materials and Methods section and in the legend to Fig. 4. After hybridization, filters were rinsed incubated for 5 min. in 2xSSC at the indicated temperature, then washed and dried as usual.

Even more surprising, DNA products made on one murine sarcoma virus RNA (Kirsten sarcoma virus carrying Gross leukemia virus) anneal only poorly to RNA from another murine virus, Rauscher leukemia virus (Table 4). That the Gross leukemia virus information is contained in the MSV DNA product is demonstrated in Table 5, wherein hybrids formed in solution were analyzed by Aspergillus nuclease treatment. Table 5 also demonstrates that Moloney leukemia virus shows little or no complementarity with MSV DNA product. The low cross-reaction observed between MSV DNA product and RLV RNA is probably not due to a low level

of identical nucleotide sequences, since the heterologous hybrid that is formed has a lower thermal stability than the homologous hybrid (Figure 3). It should be noted that considerable cross-reaction has been observed in the murine system when the hybrids are analyzed in Cs_2SO_4 gradients (data not shown). It is likely therefore, that genetic similarity exists between (e.g., leukemia) viruses, even though little or no identity is apparent.

Overall, the hybridization results presented here support the notion that RNA tumor virus information is encoded in "normal" cells and suggests that several different sets of these genes exist, capable of giving rise to different (leukemia) viruses.

ii. On the expression of the provirus

To transfer information from a DNA provirus to mature viral RNA, transcription must be required at some stage. Moreover, since the virus RNA contains poly(A) (Lai and Duesberg, 1972; Gillespie, Marshall, and Gallo, 1972; Green and Cartas, 1972) its addition to the RNA must also occur. This RNA may also be used as a template for protein synthesis during viral replication (Siegert et al., 1972). though experiments to demonstrate synthesis of specific viral proteins directed by viral RNA in vivo have not been reported.

Cells which produce no virus but which carry viral information are known to exist (nonproducer cells). These cells can be induced to produce virus particles by the addition of pyrimidine analogues such as iododeoxyuridine (IDU) and bromodeoxyuridine (BrDU) as demonstrated by Lowy et al., (1972) and Aaronson et al. (1972). These authors have also demonstrated a similar induction of C-type virus from presumably uninfected cells. In the above context, a tentative scheme of viral replication is diagrammatically illustrated in Figure 4.

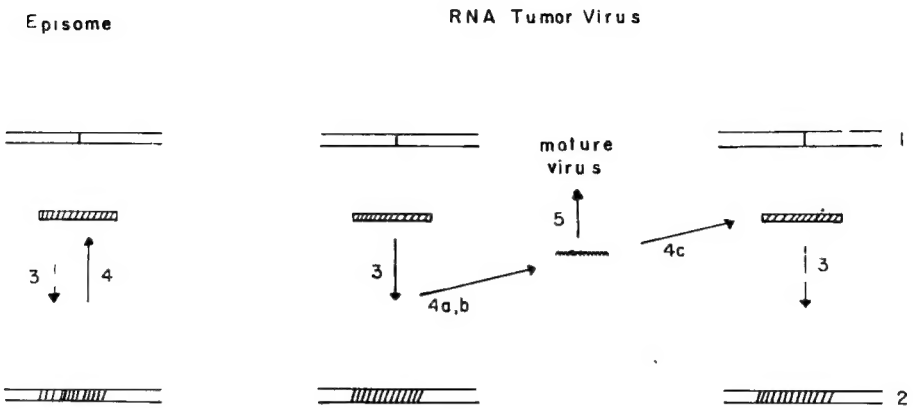

Fig. 4. Schematic comparison of episomal and RNA tumor virus systems. Shaded symbols = episomal or proviral genetic information, unshaded symbols = the remaining cell genome. 1 = the autonomous episomal state, 2 = the integrated state. 3 = the conversion from the autonomous to the integrated state. 4 = the conversion from the integrated to the autonomous state. 5 = production of mature virus particles. Steps 4a and 4b involve RNA synthesis and processing, respectively;

step 4c involves the formation of a DNA copy of (some of) the provirus transcripts.

The IDU induction procedure provides a simple system to study expression of supposed proviral genes. We focus here on the step of addition or incorporation of poly(A) into viral RNA and ask whether it is necessary for successful virus replication. Cordycepin (3'-deoxyadenosine, 3'dA) has been shown to block the addition of poly(A) sequences onto newly synthesized RNA transcripts (Darnell et al., 1971). Therefore, it was of interest to measure the effect of 3'dA on the induction of virus by IDU.

For this purpose, Balb/3T3 (uninfected) or Balb/K3T3 (nonproducer) cells were exposed to IDU (40 µg/ml) for 24 hours to induce virus production. In one experiment 3'dA was added to the culture with the IDU and was removed after 24 hrs. The relative amount of virus production was measured by assaying reverse transcriptase activity associated with virus-like particles in the culture medium. The result of this study is shown in Figure 5. Particulate enzyme activity reaches a maximum around 3-4 days after induction. 3'dA completely inhibits the appearance of this activity. The effect of cordycepin is due neither to cell killing nor to a general cytotoxic effect (Wu et al., 1972). If one assumes in the induction system a mechanism of action of 3'dA which is comparable to reported data in other systems (Darnell et al., 1971), it can be concluded that the addition of poly(A) to some RNA species is essential for the production of virus particles with reverse transcriptase activity.

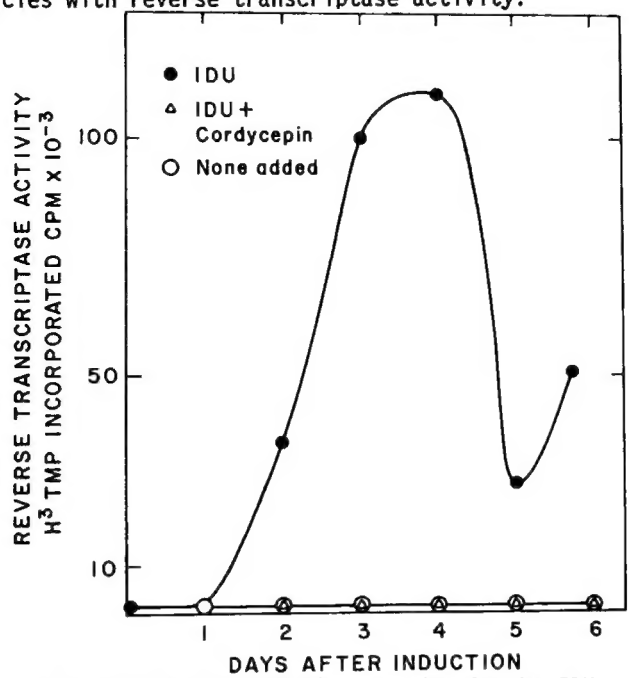

Fig. 5. Time course of induction of virus production by IDU. 10^6 cells (BALB/K3T3) were plated in 100 mm Petri dish. IDU (40 µg/ml) and/or cordycepin (100 µg/ml, Sigma Chemical Co.) were added two days after plating the cells. The cells were exposed to the compounds for 24 hours. The media were harvested, stored at -20°, and the new media was added to each plate daily. The virus particles in 10 ml of harvested media were pelleted by centrifugation and resuspended in 0.25 ml of buffer containing 1 mM Tris HCl pH 7.9, 20 mM KCl, 1 mM EDTA, 1 mM DTT and 50% glycerol. The reverse transcriptase was assayed in a reaction mixture of 100 µl containing 40 mM Tris HCl pH 7.9, 60 mM KCl, 1 mM DTT, 1.5 mM $_3$Mn acetate, 0.2 mM EDTA, 0.1% Triton, 20 µg/ml of $(dT)_{12-18}$·poly (rA), 7.2 mµM H^3 TTP (14 mCi/µmole, Schwartz Biochemical) and 20 µl of viral solution. The reaction was

carried out at 30°C for 90 minutes. The reaction was stopped by chilling and the TCA precipitable count was measured.

Table 6
Effective Time Course of IDU Induction and Cordycepin Inhibition

Period of Treatment with IDU or Cordycepin (hours)	Reverse Transcriptase Activity(cpm 10^{-3})		Period of Treatment		Reverse Transcriptase Activity(cpm 10^{-3})
	with IDU	with IDU and Cordycepin	with IDU	with Cordycepin	
0-8	110.4	not tested	0-8	8-32	1.7
0-12	220.8	199.8	0-12	12-36	1.6
0-16	297.4	1.5]	0-18	18-42	118.2
0-24	285.3	1.0	0-24	24-48	210.4

Balb/K3T3 cells were grown in the manner similar to that described in Fig. 5. They were treated with IDU (40 µg/ml) and/or cordycepin (100 µg/ml) for the time period according to the sequence indicated on the table. Media were harvested and new media were added daily. The reverse transcriptase activity associated with viral particles in media was assayed according to the procedure described in Fig. 5. Reverse transcriptase activity in the table are based on activity at the time of maximum virus production; about the third to fourth day after induction (see Fig. 5).

3'dA affects virus production at a critical, relatively short period during the induction process (Table 6). If the 3'dA is added with IDU, but washed out 12 hrs. later, no 3'dA effect is observed. Similarly, if the addition of 3'dA is delayed for 18-24 hrs. after the addition of IDU, virus production occurs normally. 3'dA dissects the viral induction process into 3 distinct phases; an early and a late 3'dA-resistant phase and an intervening 3'dA-sensitive phase. We feel that these phases represent RNA transcription, RNA processing, and RNA translation plus virus assembly. This interpretation is being tested.

iii. RNA and RNA-dependent DNA polymerase in human leukemia

Poly(A) addition seems to be critical for virus production in the murine system. Since data is emerging that virus-like particles may be associated with human cancers, it is of some importance to attempt a search in humans for RNA with similar properties to that found in animal virus systems. Progress has been reported in such a search by Spiegelman and his associates, using nucleotide sequence homology with animal virus RNA as a criterion (see Hehlman et al. and references cited therein), but confirmation by a separate technique is critical.

The RNA of RNA tumor viruses in well-defined animal systems is a 60-70S aggregate which contains large stretches of poly(A) (Gillespie, Marshall, and Gallo, 1972). In the assay system used, that of molecular hybridization of the test RNA with radioactive poly(U) followed by appropriate nuclease treatment,the poly(A) regions migrate as a relatively homogeneous peak on polyacrylamide gels, compared to other RNA species, such as mouse liver total RNA, rabbit reticulocyte hemoglobin mRNA, or EMC RNA (Gillespie et al., 1972). RNA with the same properties has been found in less defined systems; in the Mason-Pfizer agent (Gillespie, Marshall, and Gallo, 1972) in visna virus (Gillespie et al., 1972), and in some human milks (Schlom et al., 1972).

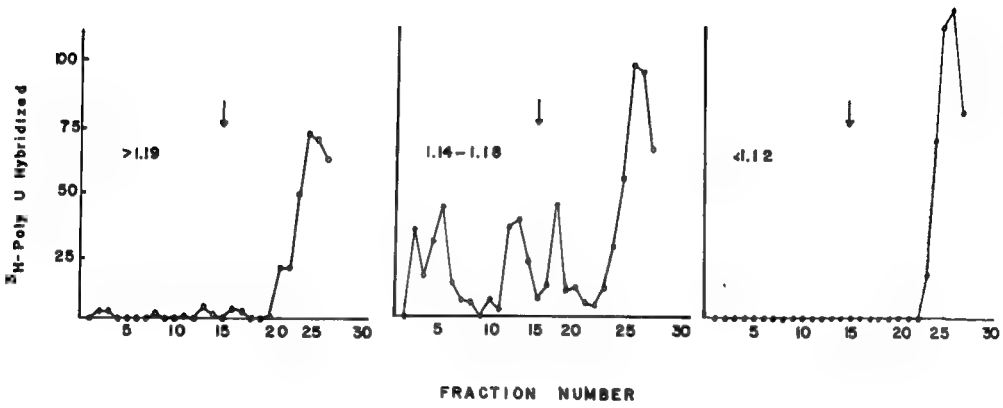

Fig. 6. Analysis of RNA in the cytoplasmic pellet fraction of leukemic cells. See Materials and Methods for procedural details. RNA was extracted from isopycnically banded particulate cytoplasmic isolates, then fractionated in sucrose density gradients. ^{3}H poly(U) was hybridized to an aliquot of each fraction (Gillespie, Marshall, and Gallo, 1972). Ordinate = cpm. Arrow indicates position of 60-70S RNA. Density of banded material is indicated in each panel.

Figure 6 shows that the situation is again encountered in RNA isolated from a particulate subcellular fraction of peripheral blood lymphocytes donated by leukemic patients. 70S poly(A)-containing RNA is found in a particulate cytoplasmic fraction of disrupted cells. Other subcellular fractions have not been analyzed in detail, because of overlap arising from heterogeneous nuclear RNA. In order to obtain reproducible results, the particulate fraction had first to be banded isopycnically in sucrose gradients prior to RNA isolation. When this procedure was carried out (on 4 separate cell lots), 3 showed 70S poly(A)-containing RNA in only the 1.16 density fraction while one exhibited this RNA only in a higher density fraction. In every case examined, the 70S material was destroyed by alkali treatment.

The yield of this RNA is inordinately low, prohibiting necessary structural studies. However, in cells of one patient, the RNA was recovered in reasonable quantity from an unbanded cytoplasmic particulate fraction and this RNA: 1) was contained in a complex which protected it from mild RNAase treatment before phenol extraction, 2) "dissociated" to low molecular weight material (including a 35-40S entity) when exposed to 55° in low salt, and 3) had a long, homogeneous stretch of poly(A). Thus, the properties of this RNA species encourage further analysis, but at this point should not be used to indicate a viral etiology for human leukemia.

A second approach toward establishing the presence of viral related information in human leukemia involves the analysis of RNA dependent DNA polymerase. The presence of reverse transcriptase in RNA tumor viruses has been extensively reviewed (Gallo, 1971; Temin and Baltimore, 1972). A similar activity was described in human acute leukemic blood lymphoblasts (Gallo et al., 1970, 1971). Many laboratories subsequently reported the detection of reverse transcriptase activity in extracts from normal cells (Scolnick et al., 1971; Penner et al., 1971). based on their response to poly (dT)·poly (rA) as a template-primer. Poly (dT).poly (rA) has since been shown to be nonspecific (Baltimore and Smoler, 1971; Goodman and Spiegelman, 1971; Robert et al., 1972; Wells et al., 1972; Smith and Gallo, 1972).

More recently, we have been able to show the presence of an endogenous ribonuclease sensitive activity in the high speed pellets from patients with acute leukemia (Sarngadharan et al., 1972; Gallo et al., 1972 b). This enzyme activity purified from the pellet behaves like a true reverse transcriptase (see below, and Sarngadharan et al., 1972; Gallo et al., 1972 b). The purified leukemic enzyme is able to transcribe heteropolymeric portion of 70S RNA from avian myeloblastosis virus (AMV) and in addition, prefers $(dT)_{12}$.poly (rA) over $(dT)_{12}$.poly (dA) as a template-primer (in the presence of Mg^{++}; in the presence of Mn^{++} we find that the major normal cellular DNA polymerases also show preference for $(dT)_{12}$.poly (rA). Leukocytes obtained from the peripheral blood of patients with acute leukemia were first washed with phosphate buffer. The cells were then disrupted by dounce homogenization in phosphate buffer, and centrifuged at 60,000 x g for 60 minutes to give a cytoplasmic pellet. The cytoplasmic pellet was suspended in 0.1 M Tris-HCl (pH 8.3) containing 0.001 M DTT and 0.2% Triton X-100. A small sample was centrifuged on 20-60% glycerol gradient in SW41 rotor for 3 hours at 40,000 RPM. Fractions were assayed for endogenous DNA polymerase activity with and without ribonuclease. Results of a typical experiment are shown in Figure 7. A peak of ribonuclease sensitive endogenous DNA polymerase activity was found at a density of 1.1 g/ml. The ribonuclease sensitivity of this endogenous DNA polymerase activity suggests the involvement of an RNA species.

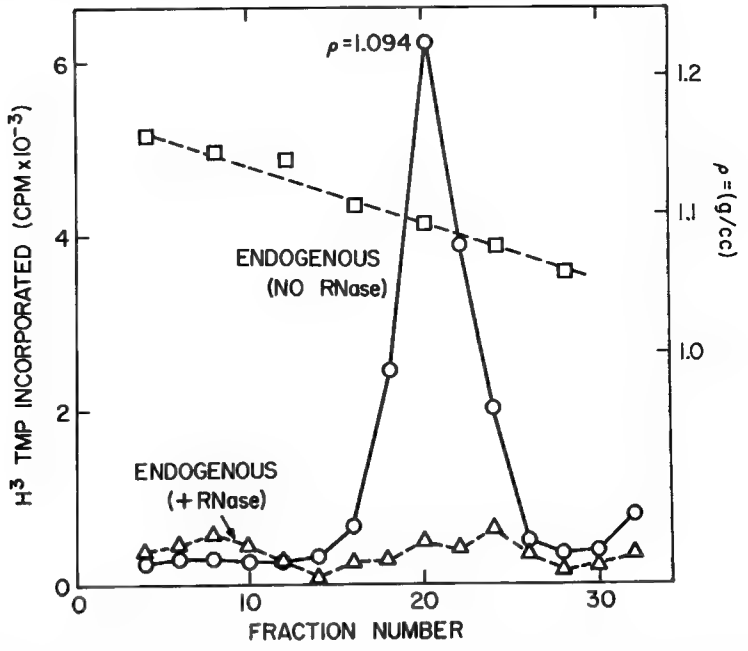

Fig. 7. Ribonuclease sensitive endogenous DNA polymerase activity of high speed cytoplasmic pellet from peripheral blood leukocytes of a patient with acute leukemia. The pellet was centrifuged (Sarngadharan et al., 1972; Gallo et al., 1972 b) on 20-60% glycerol gradient in 1 mM Tris-HCl (pH 7.9) buffer containing 1 mM EDTA and 1 mM DTT for 3 hours at 40,000 RPM in an SW 41 rotor. Fractions (0.3 ml) were collected and the endogenous DNA polymerase activity measured in 15 µl aliquots which were preincubated at room temperature for 1 hour with 0.2 M NaCl, plus RNAse (20 µg/ml) (Δ) or minus RNAse (O). Samples were incubated at 37°C for 1 hour in the standard reaction mixture (0.05 ml) containing 50 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 5 mM DTT, 50 µM each of dATP, dDTP, and dGTP, and 2.4 µM ^{3}H-TTP (8,900 cpm/pmole). The reaction was stopped by the addition of 50 µg of yeast tRNA and 2 ml of 10% trichloracetic acid containing 0.02 M sodium pyrophosphate, collected on millipore filters and counted.

If an RNA species is involved in the endogenous DNA polymerase activity of the cytoplasmic pellet, than an RNA-DNA hybrid should be detectable in the early periods of DNA polymerase reaction. In Figure 8, we show an analysis of the endogenous DNA polymerase reaction products of cytoplasmic pellets from acute lymphatic and acute myeloblastic leukemic cells. The newly synthesized DNA product from both AML and ALL cells band in the RNA and RNA-DNA hybrid density regions. When the DNA product is treated with 0.3 N KOH (to destroy RNA) and then analyzed on cesium sulfate gradients, the bands in the RNA and RNA-DNA hybrid regions disappear, suggesting that at least part of the newly synthesized DNA is attached to RNA. Moreover, the alkali treated endogenous DNA polymerase reaction product hybridizes to (5-10%) the RNA isolated from the high speed cytoplasmic pellets (data not shown). However, we feel that a latter experiment of this type does not in itself justify the conclusion that the reaction is RNA-directed.

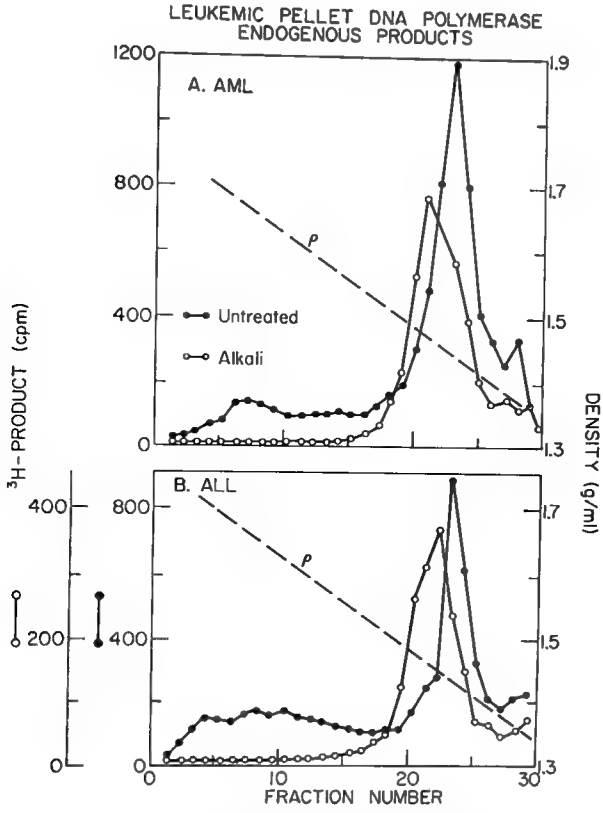

Fig. 8. Analysis of the DNA product obtained from the cytoplasmic pellet endogenous DNA polymerase reaction. Cesium sulfate equilibrium density centrifugation. Panel A, (AML, acute myeloblastic leukemia) the product analysis of an endogenous (RNAse sensitive) DNA polymerase reaction; (a) untreated (•), (b) treated with alkali (o); and Panel B, (ALL, acute lymphoblastic leukemia), (a) untreated (•), and (b) treated with alkali (o).

To further establish that the leukemic enzyme can utilize RNA as a template to carry out a DNA synthesis like the reverse transcriptase of RNA tumor viruses, we purified the enzyme from the high speed pellet. A detailed procedure for the purification of this enzyme is given elsewhere (Sarngadharan et al., 1972; Gallo et al., 1972 b). Briefly, the enzyme is extracted from the cytoplasmic pellet with salt and Triton X-100 followed by purification by DEAE cellulose, phosphocellulose and Sephadex G-200 column chromatography. The purified enzyme is able to utilize viral 70S RNA as a template to give a DNA product which preferentially hybridized back to the template (Sarngadharan et al., 1972). Figure 9 shows the analysis of the RLV 70S RNA-directed reaction product on cesium sulfate gradients, and its ability ot hybridize back with RLV 70S RNA (J. Bhattacharrya, P. Sarin, M. Xuma, M. Reitz, and R. Gallo).

Table 7

Template Characteristics of Leukemic Reverse Transcriptase

Template-Primer	pmole ^{3}H-TMP incorporated per 10 µg enzyme		
	Patient 1	Patient 2	Patient 3
$(dT)_{12-18}$·poly (rA)	71.9	22.0	446.0
$(dT)_{12-18}$·poly (dA)	56.4	5.5	89.0
Poly(dT).poly(rA)	45.7	104.8	1,219.0
Poly(dA-T)	265.7	N. T.	N. T.
$(dG)_{12-18}$·poly (rC)	6.7 (H^3 dGMP incorporated)	N. T.	N. T.

Purified enzyme was incubated with the indicated template-primer (50 µg/ml) at 37°C, for 30 minutes under conditions described in Figure 7. Poly d(AT), poly(dT).poly(rA), and poly(dA) were obtained from Miles Laboratory, $(dT)_{12-18}$·poly(rA), $(dG)_{12-18}$.poly(rC), and $(dT)_{12-18}$ from Collaborative Research, Incorporated. The conditions for the preparations of $(dT)_{12-18}$.poly(dA) are described elsewhere (Robert et al., 1972).

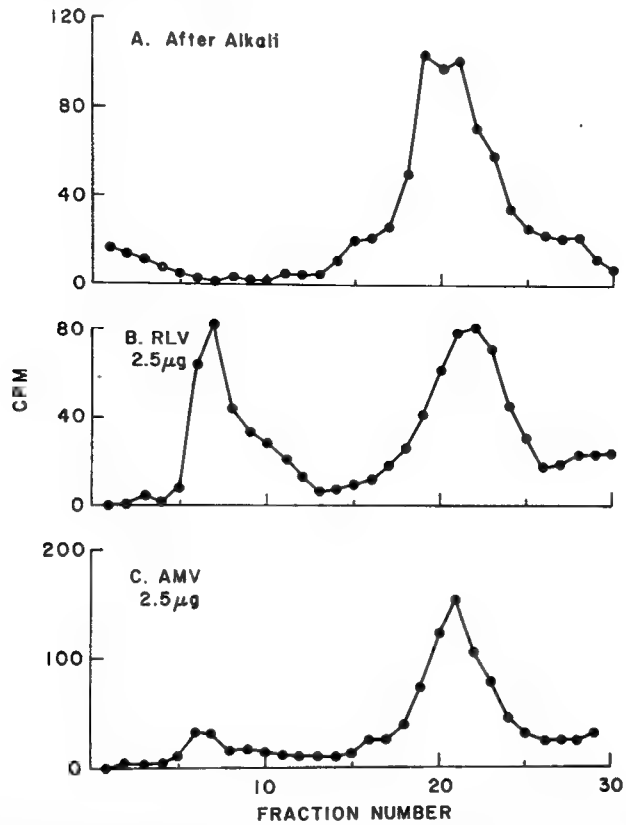

Fig. 9. Complementarity between DNA product prepared with the leukemic enzyme and RLV 70S RNA as template. The DNA product was prepared (in the absence of oligo [dT]) as described earlier (Sarngadharan et al., 1972), purified by precipitation with cetyltrimethylammonium bromide (Reitz, M. S., J. W. Abrell, C. D. Trainor, and R. C. Gallo, Biochem. Biophys. Res. Commun. 49, 30 [1972]), alkali-treated (0.3 N KOH, 95°, 10 minutes), and neutralized. This was then analyzed on cesium sulfate gradients: A) without further treatment, B) after annelaing with 2.5 µg RLV 70S RNA in 3 x SSC, 50% formamide for 72 hours at room temperature, and C) after annealing with 2.5 µg AMV 70S RNA as above.

The response of the leukemic enzyme to various synthetic DNA and RNA-DNA hybrid template-primers is summarized in Table 7. As is evident from this table, the leukemic enzyme prefers $(dT)_{12-18}$.poly (rA) over $(dT)_{12-18}$.poly (dA) and uses $(dG)_{12-18}$.poly (rC) as a template primer (incorporating ^{3}H-dGMP). DNA polymerases purified from human normal blood lymphocytes show a marked preference for $(dT)_{12-18}$.poly (dA) over $(dT)_{12-18}$.poly (rA) (10-30:1) (Smith and Gallo, 1972; Bobrow et al., 1972; Robert et al., 1972).

The leukemic enzyme is a true DNA polymerase because (a) it incorporated all four deoxynucleoside triphosphates and omission of a deoxynucleoside triphosphate results in significant loss in activity, (b) the enzyme does nct

utilize oligodeoxynucleotides as a primer for the incorporation of ^{3}H-TMP, as observed by Baltimore (personal communications) for the terminal transferase activity isolated from cells from a patient with acute lymphocytic leukemia, (c) a 3S to 5S DNA is synthesized, and (d) the DNA hybridizes back to the template RNA.

DISCUSSION

Presumptive uninfected cells have the coding potential for 10-30% of mature 70S virus RNA information (minimum) judged by hybridization assays. In our experience, the uninfected cell information is repeated at least 50-100 times. However, these data do not indicate whether these nucleotide sequences are potential translational templates since hemoglobin mRNA molecules may be coded by unique genes (Bishop, et al., 1972; Harrison et al., 1972), while histone mRNA appears to be coded by repeated genes. Finally, it is not known whether these sequences remain constant during the prolonged propagation of the virus or whether they may change when, for example, the virus infects a new host. If rapid genetic change is occurring, the use of molecular hybridization for determining the origin of viruses will have to be carried out with extreme caution, for it will be important to know whether the assay determines the immediate or original host. Our data with respect to RD114, for example, establishes that at least some of the viral information is of cat origin, but does not exclude the possibility that there is also a human component which is coded for by unique genes. This possibility is being tested directly. It should be noted that it cannot be adequately tested with hybridization probes containing only a limited fraction of the viral information.

A second aspect of the genetic information of RNA tumor virus concerns its variability in different strains of viruses which produce the same general disease. Our attempts to evaluate this have employed a survey of the leukemia virus information limited to and found in DNA products synthesized in vitro using the viral RNA as template. The data show clearly that, with respect to these nucleotide sequences, each leukemia virus is unique. Some similarity exists, since cross hybridization is observed in heterologous annealings when hybrid formation is analyzed by cesium sulfate isopycnic centrifugation, but identities do not exist when hybrid formation is assayed under the more stringent conditions reported here. It will be important to establish whether these differences arise because the viruses have independent genetic origin or whether they have originated from identical information, then undergone rapid genetic change.

Tumor virus RNA molecules contain poly(A) (Lai and Duesberg, 1972; Gillespie, Marshall, and Gallo, 1972; Green and Cartas, 1972). The data presented here show that induction of tumor viruses by iododeoxyuridine can be blocked by an adenine anologue, 3' deoxyadenosine. This analogue acts in other systems by selectively inhibiting poly(A) addition onto RNA. The possibility that addition of poly(A) to viral-specific RNA is required for the production of active virus particles is under investigation.

It is reasonable at this stage to ask whether parameters of RNA virus infection in animal systems are reflected in human neoplasia. To date, RNA and reverse transcriptase have been used as viral markers in neoplastic systems. RNA in human neoplastic cells contains a population having a partial nucleotide sequence which is similar to that found in the genome of murine RNA tumor viruses (Hehlman and Spiegelman, 1972).

Though this result has been difficult ot repeat in other laboratories, our data with the animal systems suggests that one need not expect complete identity (human vs. murine), so the extent of cross hybridization observed in any given case could be a function of the virus used to make the molecular probe and of the degree of similarity between the viral RNA and the human RNA. Experiments using other physical properties of the RNA as marker -- its 70S sedimentation value and poly(A) content -- have yielded a virus-like RNA molecule

in lymphocytes from leukemic patients (this communication) and in human milk (Schlom et al., 1972), but the low yield of this material has precluded an analysis of its nucleotide sequence. Even though this molecule has not yet been observed in normal human blood lymphocytes (data not shown), it is still possible that a more thorough search will reveal it there also. Further analysis of these cells and embryonic tissues is now in progress.

The current state of affairs with the RNA dependent DNA polymerase is based on more definitive data. The discovery and characterization of reverse transcriptase in RNA tumor viruses, and the indirect evidence supporting a biological role in the transcription of viral RNA information into a DNA transcript provide an impetus for a search for such an enzyme activity in cells which are, A) not producing virus, B) producing low levels of complete virus particles, or C) producing incomplete virus particles. Viral reverse transcriptase has been shown to be distinct from normal cellular DNA polymerases (Robert et al., 1972; Goodman and Spiegelman, 1971).

The enzyme found in human leukemic cells is distinguished from the two major normal lymphocyte DNA polymerases and has characteristics very similar to the viral reverse transcriptase. Mixing experiments (leukemic pellet polymerase with DNA polymerase from normal human lymphocytes) indicate that the failure of these enzymes to transcribe viral RNA is not due to the presence of inhibitor(s). Moreover, this enzyme is clearly a true DNA polymerase and not a terminal addition enzyme like that recently found in these cells (D. Baltimore, personal communication) since: a) all four deoxynucleotides are incorporated in relatively equal proportion, b) omission of any deoxynucleotide results in a significant reduction of activity, c) the newly synthesized DNA is 3S to 5S in size, and d) the DNA back hybridizes to its RNA template.

Is this polymerase viral or cellular in origin? A definitive conclusion on the origin of the enzyme cannot be made by biochemical criteria alone. We can state that it has the biochemical properties of oncornaviruses reverse transcriptase, but a definite conclusion on the origin of this enzyme cannot be made by biochemical criteria alone. However, experiments carried out in collaboration with Dr. G. Todaro and his colleagues (see page , this volume) have demonstrated that the human reverse transcriptases we have isolated have antigenic sites closely related to those found on primate C-type virus reverse transcriptase. The results taken together strongly argue that the human enzyme is derived from C-type virus information.

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REFERENCES

Aaronson, S. A., G. J. Todaro and E. M. Scolnick, (1971) Induction of murine C-type viruses from clonal lines of virus-free BALB/3T3 cells, Science 174,157.
Baltimore, D. and D. Smoler, (1971) Primer requirement and template specificity of the DNA polymerase of RNA tumor viruses, Proc. Nat. Acad. Sci. USA 68, 1507.
Baluda, M. A., (1972) Widespread presence in chicken of DNA complementary to the RNA genome of avian leukosis viruses, Proc. Nat. Acad. Sci. USA 69, 576.
Baluda, M. A. and D. P. Nayak, (1970) DNA complementary to viral RNA in leukemic cells induced by avian myeloblastosis virus, Proc. Nat. Acad. Sci. USA 66, 329.
Bishop, J. O., R. Pemberton and C. Baglioni, (1972) Reiteration frequency of hemoglobin genes in the duck, Nature New Biology 235, 231.
Bobrow, S. N., R. G. Smith, M. S. Reitz and R. C. Gallo, (1972) Ribonuclease-sensitive DNA synthesis in a cytoplasmic pellet of normal human blood lymphocytes, Proc. Nat. Acad. Sci. USA, in press.
Bonner, J., G. Kung and I. Bekhov, (1967) A Method for the Hybridization of Nucleic Acid Molecules at low temperature, Biochemistry 6, 3650.
Britten, R. J. and D. E. Kohne, (1968) Repeated sequences in DNA, Science 161,529.
Brown, R. D. and G. P. Tocchini-Valentini, (1972) On the role of RNA in gene amplification, Proc. Nat. Acad. Sci. USA 69, 1746.
Darnell, J. E., L. Philipson, R. Wall and M. Adesnik, (1971) Polyadenylic Acid Sequences: Role in Conversion of Nuclear RNA into Messenger RNA, Science 174, 507.
Gallo, R. C., (1971) Reverse transcriptase, the DNA polymerase of oncogenic RNA viruses, Nature 234, 194.
Gallo, R. C., S. S. Yang, and R. C. Ting, (1970) RNA dependent DNA polymerase of human acute leukemic cells, Nature 228, 927.
Gallo, R. C., S. S. Yang, R. G. Smith, R. Herrera, R. C. Ting, S. N. Bobrow, C. Davis and S. Fumioka, (1971) RNA and DNA dependent AND polymerase of human normal and leukemic cells, Second Lepetit Colloqium on Biology and Medicine, the Biology of oncogenic viruses, ed. L. Silverstri (Holland, Amsterdam) p. 210.
Gallo, R. C., P. S. Sarin and J. Bhattacharyya, (1972a) Distribution of the RNA dependent DNA polymerase in human acute leukemic cells, Cancer, in press.
Gallo, R. C., P. S. Sarin, R. G. Smith, S. N. Bobrow, M. G. Sarngadharan, M. S. Reitz and J. W. Abrell, (1972b) RNA directed and primed DNA polymerase activities in tumor viruses and human lymphocytes, in: DNA synthesis in vitro, Second Steenbock Symposium, in press.
Gelb, L., S. A. Aaronson and M. Martin, (1971) Heterogeneity of murine leukemia virus in vitro DNA; detection of viral DNA in mammalian cells, Science 174,1353.
Gillespie, D. and S. Spiegelman, (1965) A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane, J. Mol. Biol. 12, 829.
Gillespie, D., (1968) Formation and detection of DNA-RNA hybrids, Methods in Enzymol. 12B, 641.
Gillespie, S. and D. Gillespie, (1971) RNA-DNA hybridization in aqueous solution and in solution containing formamide, Biochem. J. 125, 481.
Gillespie, D., S. Marshall and R. C. Gallo, (1972) Tumor virus RNA contains poly(A), Nature New Biology 236 227.
Gillespie, D., K. Takemoto, M. Robert and R. C. Gallo, (1972) Poly(A) in visna virus RNA, Science, in press.
Goodman, N. C. and S. Spiegelman, (1971) Distinguishing reverse transcriptase of an RNA tumor virus from other known DNA polymerases, Proc. Nat. Acad. Sci. USA 68, 2203.
Green, M. and M. Cartas, (1972) The genome of RNA tumor viruses contains poly-adenylic acid sequences, Proc. Nat. Acad. Sci. USA 69, 791.
Harel, L., J. Harel and G. Frezouls, (1972) DNA copies of viral RNA in rat cells transformed by Rous sarcoma virus, Biochem. Biophys. Res. Commun. 48, 796.
Harrison, P. R., A. Hell, G. D. Birnie and J. Paul, (1972) Evidence for single copies of globin genes in the mouse genome, Nature 239, 219.
Hehlman, R., D. Kufe and S. Spiegelman, (1972) Viral related RNA in Hodgkins disease and other human lymphomas, Proc. Nat. Acad. Sci. USA 69, 1727.

Huebner, R. J. and G. J. Todaro, (1969) Oncogenes of RNA tumor viruses as determinants of cancer, Proc. Nat. Acad. Sci. USA 64, 1087.
Lai, M. M. C. and P. H. Duesberg, (1972) Adenylic Acid-Rich Sequences in RNA of Rous Sarcoma Virus and Rauscher Mouse Leukemia Virus, Nature, 235, 383.
Lowy, D. R., W. P. Rowe, N. Teich and J. W. Hartley, (1971) Murine leukemia virus: High-frequency activation in vitro by 5-iododeoxyuridine and 5-bromodeoxyuridine, Science 174, 155.
McAllister, R. M., M. Nicolson, M. B. Gardner, R. W. Rongey, S. Rasheed, P. S. Sarma, R. J. Huebner, M. Hatanaka, S. Oroszlan, R. V. Gilden, A. Kabigting, and L. I. Vernon, (1972) C-type virus released from cultured human rhabdomyosarcoma cells, Nature New Biology 235, 3.
Penman, S., (1969) Preparation of purified nuclei and nucleoli from mammalian cells, in: Fundamental techniques in virology, ed. Habel and Salzman, Academic Press (New York) p. 35.
Penner, P. E., L. H. Cohen and L. A. Loeb, (1971) RNA dependent DNA polymerase activity in mammalian cells, Nature New Biology 232, 58.
Perry, R. P., J. LaTorre, D. E. Kelley and J. R. Greenberg, (1972) On the lability of poly(A) sequences during extraction of messenger RNA from polyribosomes, Biochem. Biophys. Acta. 262, 220.
Reitz, M., D. Gillespie, W. C. Saxinger, M. Robert and R. C. Gallo, (1972) Poly(A) tracts of tumor virus 70S RNA are not transcribed in endogenous or reconstituted reactions of viral reverse transcriptase, Biochem. Biophys. Res. Comm., in press.
Robert, M., R. G. Smith, R. C. Gallo, P. S. Sarin and J. W. Abrell, (1972) Viral and cellular DNA polymerase: Comparison of activities with synthetic and natural RNA templates, Science 176, 798.
Rosenthal, P. N., H. L. Robinson, W. S. Robinson, T. Hanafusa and H. Hanafusa, (1971) DNA in uninfected and virus-infected cells complementary to avian tumor virus RNA, Proc. Nat. Acad. Sci. USA 68, 2336.
Sarngadharan, M. G., P. S. Sarin, M. S. Reitz and R. C. Gallo, (1972) Reverse transcriptase activity of human acute leukemic cells: Purification of the enzyme, response to AMV 70S RNA, and characterization of the DNA product, Nature New Biology, in press.
Saxinger, W. C., C. Ponnamperuma and D. Gillespie, (1972) Nucleic acid hybridization with RNA immobilized on filter paper, Proc. Nat. Acad. Sci. USA 69, 2975.
Schlom, J., D. Colcher, S. Spiegelman, S. Gillespie and D. Gillespie, (1972) Quantitation of RNA tumor virus and virus-like particles in human milk by hybridization to poly(A) sequences, Science, in press.
Scolnick, E. M., S. A. Aaronson, G. J. Todaro and W. P. Parks, (1971) RNA dependent DNA polymerase activity in mammalian cells, Nature 229, 318.
Siegert, W., R. N. H. Konigs, H. Bauer and P. H. Hofschneider, (1972) Translation of avian myeloblastosis virus RNA in a cell free lysate of Escherichia coli, Proc. Nat. Acad. Sci. USA 69, 888.
Smith, R. G., and R. C. Gallo, (1972) DNA dependent DNA polymerase I and II from normal human blood lymphocytes, Proc. Nat. Acad. Sci. USA 69, 2879.
Temin, H. M., (1964) Homology between RNA from Rous sarcoma virus and DNA from Rous Sarcoma virus-infected cells, Proc. Nat. Acad. Sci. USA 52, 323.
Temin, H. M. and D. Baltimore, (1972) RNA directed DNA synthesis and RNA tumor viruses, Adv. Virus Res., in press.
Varmus, H. E., R. A. Weiss, R. R. Friis, W. Levinson and J. M. Bishop, (1972) Detection of avian tumor virus-specific nucleotide sequences in avian cell DNAs, Proc. Nat. Acad. Sci. USA 69, 20.
Varmus, H. E., J. M. Bishop, R. C. Nowinski and N. H. Sarkar, (1972) Mammary tumor virus-specific nucleotide sequences in mouse DNA, Nature New Biology 238, 189.
Wells, R. D., R. M. Flugel, J. E. Larson, P. F. Schendel and R. W. Sweet, (1972) Comparison of some reactions catalyzed by DNA polymerase from avian myeloblastosis virus, Escherichia coli, and Micrococcus leuteus, Biochem. 11, 621.
Wu, A. M., R. C. Ting and R. C. Gallo, (1972a) Requirement of viral reverse transcriptase for viral induced leukemia in mice, Proc. Nat. Acad. Sci. USA,

submitted for publication.
Wu, A. M., R. C. Ting, M. Paran and R. C. Gallo, (1972b) Cordycepin inhibits induction of murine leukovirus production, Proc. Nat. Acad. Sci. USA, in press.